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Original Article

Interleukin-4 administration or zinc supplementation improves changes in effector T cells of spleen in zinc deficiency

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Summary

Nutritional zinc deficiency leads to immune dysfunction. In our previous study, we found that the number of T-helper 2 (Th2) cells in the spleen of zinc-deficient rats was reduced, and this change was improved by interleukin (IL)-4 administration and zinc supplementation. In this study, the effects of zinc deficiency on other effector T cells (Th1, Th17, and regulatory T cells) in the spleen of rats were investigated. In addition, the effects of IL-4 administration and zinc supplementation of zinc-deficient rats were determined. Five-week-old male Sprague-Dawley rats were fed a standard or zinc-deficient diet (n = 7 each) with saline or IL-4 treatment, or a zinc-deficient diet for 6 weeks, followed by a standard diet for 4 weeks. The numbers of interferon-gamma$^+$ CD4$^+$, CD161$^+$ CD3$^-$, IL-17$^+$ CD4$^+$, and IL-17$^+$ cells in the spleen of zinc-deficient rats were significantly increased compared to those in the other groups. However, the numbers of viable, IL-10$^+$, and FoxP3$^+$ CD4$^+$ cells, determined using the Viobility 405/452 Fixable Dye, in the spleen were comparable in all groups. In conclusion, spleen cells in zinc-deficient rats were induced to differentiate into Th1 and Th17 cells associated with the inflammatory response. IL-4 administration or zinc supplementation of zinc-deficient rats reduced the numbers of Th1 and Th17 cells, which are inflammation-related factors.

Introduction

Zinc is an essential trace element in humans and animals[1-3)]. The recommend daily zinc intakes for men and women older than 20 years are 11 and 8 mg in Japan, respectively[4)]. However, zinc intake has been reported to be deficient worldwide[5-8)].

Zinc deficiency is associated with various diseases such as growth retardation, taste abnormalities, dermatitis, hair loss, and inflammatory diseases[9,10)]. Our previous study showed no change in the number of CD4$^+$ T cells in the spleen following zinc deficiency[11,12)]. However, zinc deficiency decreases the concentrations of T-helper 2 (Th2) cell master transcription factor, GATA-3, and zinc finger proteins[12)]. Consequently, naïve CD4 cells do not differentiate into Th2 lymphocytes or produce interleukin (IL)-4 and IL-13. This, in turn, inhibits immature macrophages from differentiating into M2 macrophages[12)]. As M2 macrophages have anti-inflammatory functions, the inflammatory response to reactive oxygen species or infection persists[12)]. Therefore, it was suggested that the inflammatory response in zinc deficiency is due to a decrease in the Th2 cell–M2 macrophage pathway[12)]. However, it was unclear whether Th2 cells were specifically reduced by zinc deficiency or whether this deficiency affected other effector T cells. We also reported that the zinc-deficiency-induced inflammatory response was both suppressed by IL-4 administration and restored by zinc supplementation[12)]. However, it has not been clarified whether these treatments were effective against other effector T cells.

In this study, we analyzed the populations of Th1, Th17, induced regulatory T (iTreg), and natural killer (NK) cells in the spleens of rats fed a standard or zinc-deficient diet. Furthermore, we examined whether IL-4 administration or zinc supplementation for zinc deficiency affected these effector T cells.

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Methods

Animal design

Five-week-old male Sprague-Dawley rats, weighing 180–200 g, were obtained from Charles River Laboratories (Tokyo, Japan). Standard and zinc-deficient diets were specially made by Oriental Yeast (Tokyo, Japan). Animal experiments were performed as described in our previous report (Experiment II)[12]. Briefly, Sprague-Dawley rats were divided into four groups (n = 4/group): one group was fed 17 g/day of a standard diet (containing 0.01 % zinc) for 6 weeks (standard group); two groups were fed 17 g/day of a zinc-deficient diet and were injected intraperitoneally with saline (zinc deficiency group) or 100 ng IL-4 (Wako, Tokyo, Japan) dissolved in saline (zinc deficiency/IL-4 i.p. group) three times a week[13], and one group was fed a zinc-deficient diet for 6 weeks followed by a standard diet for 4 weeks (zinc deficiency/standard group). The animals were housed in separate cages at 22℃ with a 12-h light/dark cycle, in accordance with the protocols and guidelines approved by the Animal Experimentation and Ethics Committee of the Jikei University School of Medicine (2020–022). Body weights were determined twice per week. After dietary manipulation, blood samples were collected from the abdominal aorta under isoflurane anesthesia, and the spleens were harvested in 10 % neutral buffered formalin or Roswell Park Memorial Institute 1640 medium.

Serum zinc and copper concentrations

To obtain serum, blood was transferred to a 15-mL centrifuge tube and centrifuged at 1250 *g* for 10 min. Serum zinc and copper levels were quantified using the ACCURASAUTO zinc and QUICKAUTO NEO copper kits (Shino-test, Kanagawa, Japan), respectively, as described previously[14, 15].

Flow cytometry (FACS)

Splenic cell suspensions were prepared from the collected spleens using a cell strainer (BD Falcon, Tokyo, Japan)[16, 17]. Red blood cells from the splenic cell suspension and peripheral blood were removed by adding 5 mL erythrocyte lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA), incubating on ice for 1 min, and centrifuging at 180 *g* for 5 min at 4℃. Isolated splenocytes were diluted in phosphate-buffered saline (PBS) with 5 % fetal bovine serum (FBS) to obtain a cell concentration of 1×10^6 cells/mL, blocked with goat serum (Jackson ImmunoResearch, Baltimore, PA, USA) at 4℃ for 10 min, washed with PBS containing 5 % FBS, and probed with the following antibodies: AP-Foxp3 (1 : 100 dilution; BioLegend, San Diego, CA, USA), APC-CD4 (1 : 100 dilution; BioLegend), PE-CD4 (1 : 100 dilution; BioLegend), FITC-interferon (IFN)-γ (1 : 100 dilution; BioLegend), APC-IL-17 A (1 : 100 dilution; Thermo Fisher, Waltham, MA, USA), PE-CD161 (1 : 100 dilution; BioLegend), and APC-CD3 (1 : 100 dilution; BioLegend). Cell viability was assessed with the Viobility 405/452 Fixable Dye kit (Miltenyi Biotec, Auburn, CA, USA) diluted in PBS with 5 % FBS at 4℃ for 30 min. Data were collected using a MACSQuant analyzer and processed using MACSQuantify software (Miltenyi Biotec, Auburn, CA, USA).

Immunohistochemistry

Spleen sections (6-μm-thick) were analyzed immunohistochemically as described previously[18, 19]. Spleen sections were deparaffinized by serial incubation for 3 min in xylene (×3), 99.5 % ethanol (×2), 80 % ethanol (×1), and 70 % ethanol (×1). The specimens were then washed under gently running tap water for 5 min (×1) and immersed in distilled water for 1 min (×2). Endogenous peroxidases were inactivated using 0.3 % hydrogen peroxide. Spleen sections were blocked with Blocking One (Nacalai Tesque, Tokyo, Japan) for 50 min, washed with Tris-buffered saline (TBS) for 5 min (×2), and probed overnight with antibodies against IL-10 (Biorbyt, Oxford, UK) and IL-17 A (Santa Cruz Biotechnology, Dallas, TX, USA). Antibodies were diluted 1 : 50 in TBS containing 1 % bovine serum albumin. The sections were then washed with TBS for 5 min (×2), incubated with Envision™ + polymer (Dako, Tokyo, Japan) for 50 min, thoroughly washed with TBS, reacted with horseradish peroxidase-conjugated streptavidin, and stained with 0.3 % diaminobenzidine (Agilent Technologies, Santa Clara, CA, USA). Nuclei were stained with hematoxylin, and the sections were mounted using EntellanNeu (Merck-Millipore, Tokyo, Japan). To assess IL-10 and IL-17 A immunohistochemical staining, 20 randomly selected fields from the same spleen section were photographed at a magnification of 200 × using a fluorescence microscope (BZ-9000; Keyence, Tokyo, Japan). $IL\text{-}10^+$ and $IL\text{-}17\ A^+$ cells were counted and averaged from the 20 fields. To confirm the results of histological scoring objectively, computer-assisted analysis was conducted using WinROOF2018 image processing software (Mitani, Tokyo, Japan).

Statistical analyses

Data were analyzed using JMP 11.2 software (SAS Institute, Cary, NC, USA). Groups were compared using an analysis of variance followed by the Tukey–Kramer test. Differences were considered significant at $P < 0.05$.

Results

Body weights, and serum zinc and copper concentrations

Table 1 shows the final body weights, and serum zinc and copper concentrations of each group. The mean body weights of the zinc deficiency and zinc deficiency/IL-4 i.p. groups were significantly lower than those of the standard and zinc deficiency/standard groups. In addition, the body weight of the standard group was significantly increased than that zinc-deficiency/standard group. Serum zinc concentrations of the zinc deficiency and zinc deficiency/IL-4 i.p. groups were significantly lower than those in the standard and zinc deficiency/standard groups. In contrast, serum copper concentrations of the zinc deficiency and zinc deficiency/IL-4 i.p. groups were significantly higher than those in the standard and zinc deficiency/standard groups.

Th1 cells, living cells, dead cells, and NK cells

Figure 1 shows the populations of Th1 cells (IFN-γ^+ $CD4^+$), NK cells ($CD161^+$ $CD3^-$), living cells (Viobility™ Fixable $Dyes^-$), and dead cells (Viobility™ Fixable $Dyes^+$)

Table 1. Serum indices of rats fed with a standard diet or zinc-deficient diet and injected with saline (zinc deficiency) or IL-4 (zinc deficiency/ IL-4 i.p.) or subsequently switched to a standard diet (zinc deficiency/ standard).

	Standard	Zinc deficiency	Zinc deficiency/IL-4 i.p.	Zinc deficiency/standard	*P*-value
Final body weights (g)	359.3 ± 10.9	225.3 ± 6.42 ****###	231.5 ± 2.75 ****###	318.9 ± 7.40 *	<.0001
Serum zinc (μg/dL)	122.8 ± 10.3	34.17 ± 4.48 ****###	38.22 ± 7.07 ****###	130.8 ± 9.07	<.0001
Serum copper (μg/dL)	117.1 ± 6.20	198.1 ± 4.98 ****###	191.5 ± 17.2 ****###	108.4 ± 4.34	<.0001
Cu/Zn ratio	0.979 ± 0.11	6.112 ± 0.80 ***##	5.905 ± 1.67 ** #	0.845 ± 0.09	0.0014

Mean values and standard errors are indicated (n = 4 per group). $^*P < 0.05$ vs. standard; $^{**}P < 0.01$ vs. standard; $^{***}P < 0.001$ vs. standard; $^{+}P < 0.05$ vs. zinc deficiency/IL-4 i.p.; $^{++}P < 0.01$ vs. zinc deficiency/IL-4 i.p.; $^{\#}P < 0.05$ vs. zinc deficiency/standard; $^{\#\#}P < 0.01$ vs. zinc deficiency/standard; $^{\#\#\#}P < 0.001$ vs. zinc deficiency/standard by the Tukey-Kramer test.

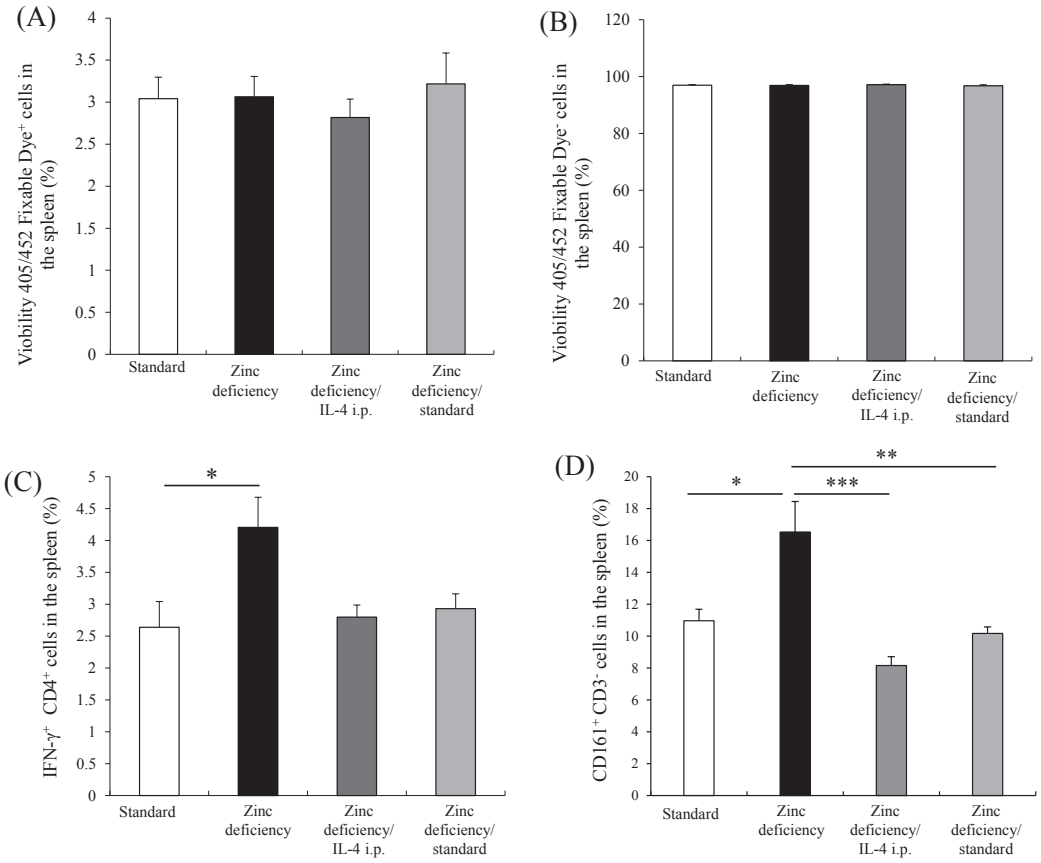

Fig. 1. Populations of interferon (IFN)-γ^+ $CD4^+$, $CD3^-CD161^+$, and Viobility 405/452 Fixable Dye stained cells in the spleen of rats. Rats were fed a standard or zinc-deficient diet and injected intraperitoneally with saline (zinc deficiency) or interleukin (IL)-4 (zinc deficiency/IL-4 i.p.), or subsequently switched to a standard diet (zinc deficiency/standard). Flow cytometry of cells stained with FITC-IFN-γ, PE-CD4, PE-CD161, APC-CD3, or the Viobility 405/452 Fixable Dye were gated on side scatter/forward scatter. (A) Positive cells by Viobility 405/452 fixable dye measured as dead cells, (B) Negative cells by Viobility 405/452 fixable dye measured as live cells. (C) Percentages of IFN-γ^+ $CD4^+$ or (D) $CD3^-CD161^+$ cells in the spleen. Data represent means ± standard errors (n = 4 per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by the Tukey-Kramer test.

in the spleens of rats. The mean percentage of Th1, and NK cells in the spleen of zinc-deficient group was significantly higher than that in all other groups. No significant change in the mean percentage of Th1 and NK cells was observed in standard, zinc deficiency, and zinc deficiency/standard groups. However, living cells, and dead cells of the spleen were comparable in all groups.

Th17 and IL-17 A$^+$ cells

Figure 2 shows the population of Th17-like cells (IL-17 A$^+$ CD4$^+$), and immunohistochemical staining for IL-17 A$^+$ cells in the spleen of rats. The mean IL-17 A positive cells and Th17-like cells in the spleen from the zinc deficiency group was significantly higher than those of the other groups. However, no significant change was observed in standard, zinc deficiency, and zinc deficiency/standard groups.

iTreg cells and IL-10$^+$ cells

Figure 3 shows the population of iTreg cells (Foxp3$^+$CD4$^+$) and immunohistochemical staining for IL-10$^+$ cells in the spleen of rats. The mean percentages of IL-10$^+$ cells and iTreg cells were unchanged in all groups.

Discussion

Essential trace elements, such as zinc, play important roles in maintaining immune function. In our previous study, we found that the spleen of zinc-deficient rats had a decreased number of Th2 cells[12]. However, the effects of IL-4 administration and zinc supplementation on other effector T cells have not been investigated. In this study, immunohistochemical staining and FACS analyses were used to examine the effects of zinc deficiency on effector T cells in the spleen of rats. In addition, the abilities of IL-4 administration and zinc supplementation to reverse these effects were observed.

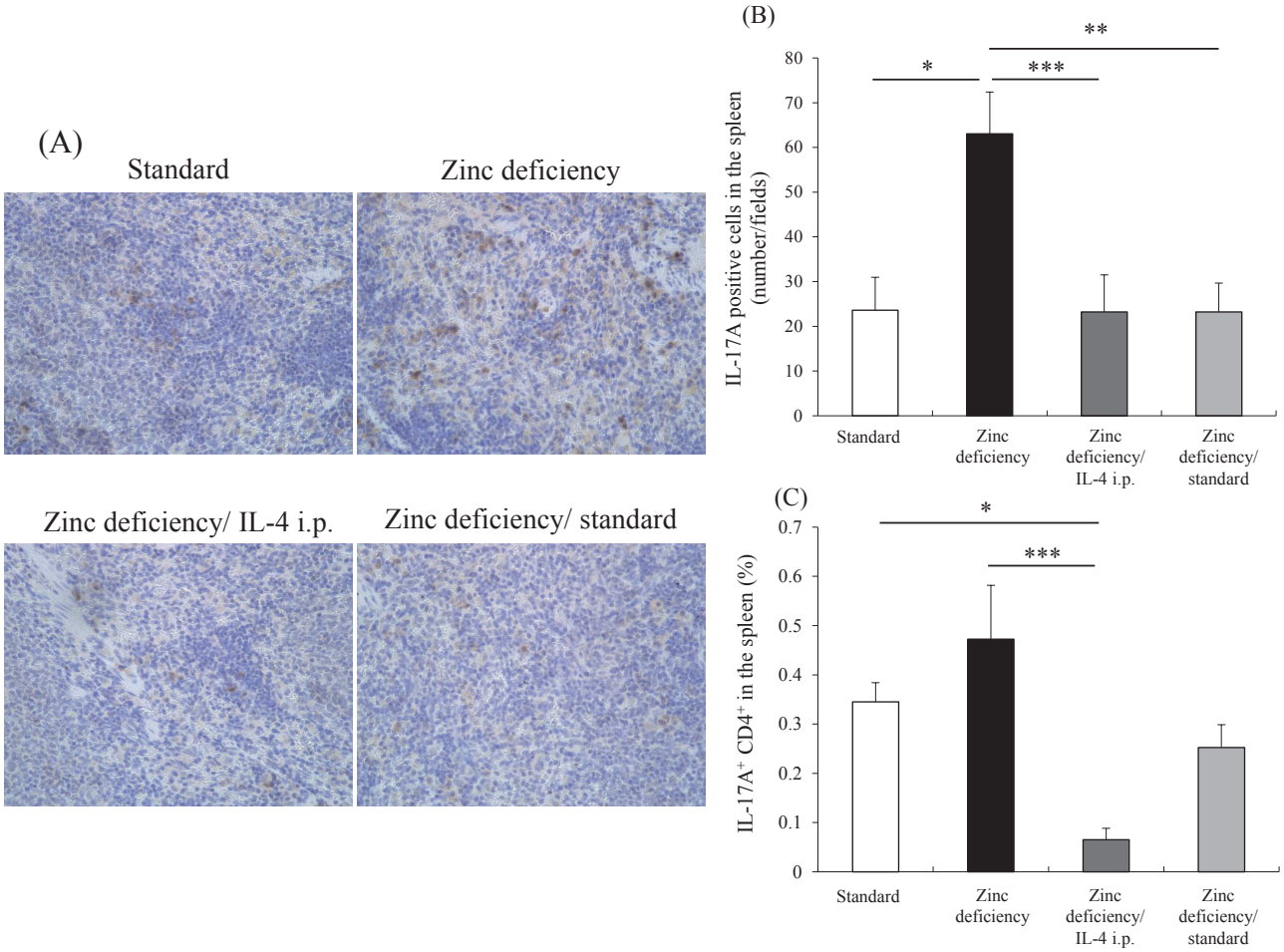

Fig. 2. Immunohistochemistry for interleukin (IL)-17A$^+$ cells and the population of IL-17A$^+$CD4$^+$ cells in the spleen of rats. Rats were fed a standard or zinc-deficient diet and injected intraperitoneally with saline (zinc deficiency) or IL-4 (zinc deficiency/IL-4 i.p.), or subsequently switched to a standard diet (zinc deficiency/standard). (A) Representative photomicrographs of immunohistochemical staining for IL-17A (a marker of Th17 cells; brown spots). (B) Percentages of IL-17A$^+$ cells per field. (C) Flow cytometry results for cells stained with APC-IL-17A and PE-CD4 antibodies and gated on side scatter/forward scatter. Data represent means ± standard errors (n = per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by the Tukey-Kramer test.

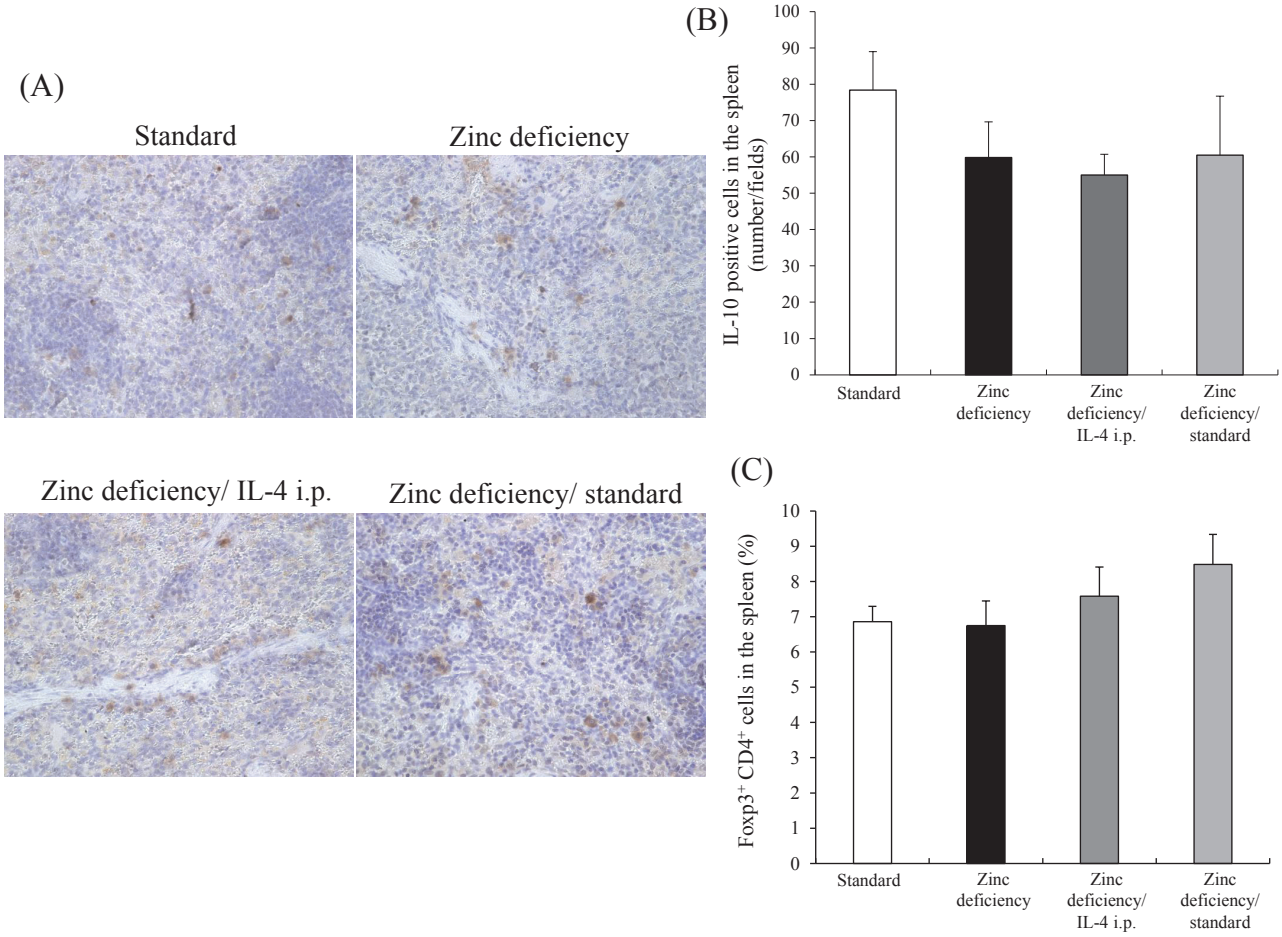

Fig. 3. Immunohistochemistry for interleukin (IL)-10$^+$ cells and the population of Foxp3$^+$CD4$^+$ cells in the spleen of rats. Rats were fed a standard or zinc-deficient diet and injected intraperitoneally with saline (zinc deficiency) or IL-4 (zinc deficiency/IL-4 i.p.), or subsequently switched to a standard diet (zinc deficiency/standard). (A) Representative photomicrographs of immunohistochemical staining for IL-10 (a cytokine that induces iTreg cells; brown spots). (B) Percentages of IL-10$^+$ cells per field. (C) Flow cytometry results for cells stained with PE-Foxp3 and APC-CD4 antibodies and gated on side scatter/forward scatter. Data represent means ± standard errors (n = 5 per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by the Tukey-Kramer test.

Compared to the other groups, the zinc deficiency and zinc deficiency/IL-4 i.p. groups showed a significant decrease in body weights and serum zinc concentrations. In contrast, weight gains in the standard and zinc deficiency/standard groups suggested that zinc plays an important role in the growth process.

Among effector T cells, the population of Th1 (IFN-γ^+CD4$^+$) cells increased in the spleen of zinc-deficient rats. In contrast, Kido et al. (2019) demonstrated that Th2 cells were decreased in the spleen of zinc-deficient rats. Therefore, it was suggested that in zinc deficiency, the Th1/Th2 relationship tends to favor Th1 cells[12]. The number of NK cells in zinc-deficient rats also increased. Th1 and NK cells also eliminate viral and bacterial infections and produce IFN-γ[21]. Previously, we observed that rats on a zinc-deficient diet for 6 weeks were more susceptible to strong dermatitis-associated infections[11,12]. Therefore, in zinc deficiency-related infections, CD4 + T cells are thought to promote differentiation into Th1 cells via the induction of IFN-γ. Furthermore, the number of Th17 cells, which produce inflammatory cytokines such as IL-17 A, was increased in the zinc deficiency group. Hence, the inflammatory response to infection is thought to induce the differentiation into Th17 cells via the induction of IL-17 A[20]. In fact, Kido (2021) showed an increase in neutrophils in the spleen of zinc-deficient rats[21]. It has also been reported that neutrophils and Th17 cells are interrelated[22]. These reports suggest that the Th17 cell–neutrophil pathway is also activated during zinc deficiency.

No significant changes were observed in iTreg cells and IL-10$^+$ cells in all groups. iTreg cells differentiate in response to IL-10. Therefore, it was considered that iTreg cells did not change because there was no change in IL-10$^+$ cells. In the future, it will be necessary to examine Treg cells derived from the thymus.

Th1, Th17, iTreg and NK cells in the zinc deficiency/IL-4 i.p. group did not increase. It is possible that increases in Th1 and Th17 cells did not occur because differenti-

ation into Th2 cells was induced by IL-4 administration. It is also possible that activation of the Th2 cell–M2 macrophage pathway suppressed the inflammatory reaction and did not increase NK cells.

Zinc supplementation improved the function of the transcription factor GATA-3 in the zinc deficiency[12]. As a result, the number of Th2 cells also increased, and it is thought that the balance of Th1, Th2, Th17, and iTreg cells was maintained.

In conclusion, our results suggested that under zinc deficiency, the Th1/Th2 balance of splenic effector T cells is altered. In addition, Th17 cells associated with the inflammatory response due to zinc deficiency also increase, suggesting its contribution to the inflammatory response (Fig. 4). Meanwhile, it was revealed that IL-4 administration or zinc supplementation under zinc deficiency improves the balance of effector T cells.

Funding

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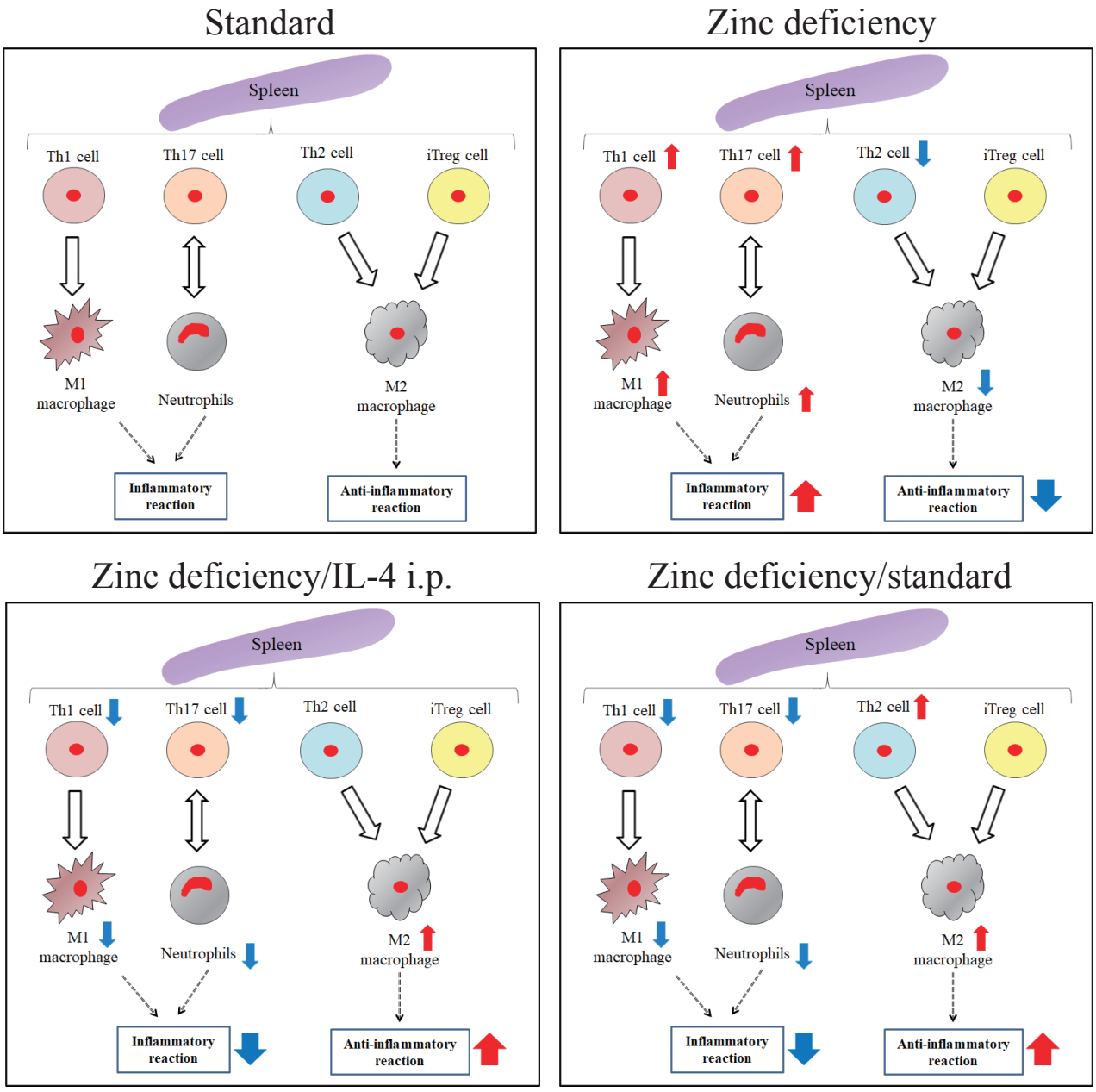

Fig. 4. Correlation diagram of the inflammatory response mediated by immunocompetent cells in the spleen due to zinc deficiency.

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